ORIGINAL

Modification of multiple ion channel functions *in vivo* by pharmacological inhibition : observation by threshold tracking and modeling

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Abstract : Maintenance of axonal excitability relies on complex balance by multiple ion currents, but its evaluation is limited by *in vitro* single channel neurophysiological study on overall behavior. We sought to evaluate behaviors of multiple ion currents by pharmacological blockade. The threshold tracking technique was used to measure multiple excitability indices on tail sensory nerve of normal male mice before and after administration of either $BaCl_2$ or ivabradine. Mathematical modeling was used to identify the interval changes of the channel parameters. After administration of $BaCl_2$ and ivabradine, the following changes were present : greater threshold changes of both depolarizing and hyperpolarizing threshold electrotonus by both ; additionally, reduced S2 accommodation, reduced late subexcitability and increased superexcitability by $BaCl_2$, increased S3 accommodation by ivabradine. Mathematical modelling implied reduction of slow K^+ conductance, along with reduction of H conductance (Ih) by $BaCl_2$; and reduction of Ih while augmentation of K^+ conductances by ivabradine. Pharmacological blockade of a selective ion channel may be compensated by other ion channels. Unintended effects by ion channel modification could be caused by secondary current alteration by multiple ion channels. J. Med. Invest. 64 : 30-38, February, 2017

Keywords **:** *axonal excitability, ion channels, membrane potential, slow K^+ channel, HCN channel*

INTRODUCTION

Maintenance of excitability of neurons and the axons is the key for proper neuronal functioning. This fact is easily recognized in disease conditions that are characterized by abnormal excitability, such as neuropathic pain, muscle cramps, and paralysis. Indeed, many factors are associated with its maintenance, namely electrolytes, ion channels, and pumps (1).

Ion channels have diverse characteristics. Channels with brief opening kinetics are important in sudden transmembrane ionic exchanges and thus are responsible for generation of an action potential (e.g., voltage-gated Na^+ channels). On the other hand, channels with slow opening and closing kinetics have little influences on the generation of action potential. Instead, they are important in setting a baseline excitability by changing the resting membrane potential (RMP). If RMP becomes depolarized (e.g., from -70 mV to -65 mV), the firing threshold becomes closer, so that even smaller transmembrane ion exchange can suffice for reaching the threshold to the generation of an action potential, resulting in extra-discharges and spontaneous firing.

Ion channels that produce subthreshold conductances are important in setting the baseline excitability and the following channels have been recognized in the peripheral axons : (1) slow K^+ current (inhibitory), (2) leak current (inhibitory), (3) hyperpolarization-activated cation current (Ih) (excitatory or inhibitory), and (4) persistent Na^+ current (excitatory) (1). To reflect the functional importance of these currents, pharmacological agents to modify these currents have shown therapeutic effects on neurological and other diseases. It is thus important to monitor interval changes of axonal excitability to achieve intended therapeutic goals by channel-modifying agents. It is highly possible that compensatory currents interfere with the intended modification of axonal excitability. Although detailed responses by ion-current modifying agents are observable *in vitro* such as patch clamping, the whole *in vivo* effects may not be easily assessed by such techniques. Threshold tracking assesses axonal excitability *in vivo*, thus a preferred method to identify interval changes of particular currents/channels by pharmacological intervention (2).

The aim of the present study was to assess the overall axonal excitability and potential compensatory mechanisms in a peripheral nerve *in vivo* by pharmacological modification.

METHODS

Study protocol. The experiment was approved by the local animal facility in Tokushima University. ICR male mice (SLC, Hamamatsu, Japan), 7-8 week-old were tested. The following anesthetic protocol was used for electrophysiological testing. Isoflurane (2%) was mixed with oxygen (1.5 l/min) for induction in a plastic box. Within 3 minutes after induction, the mouse became sedated and was transferred to an exam table where isoflurane (1.5%) was provided through nasal tube throughout the excitability testing. The animal was adjusted for 30 minutes before the electrophysiological study was initiated. The body temperature was maintained above 33°C by a thermostat-regulated heating pad.

Key points
- Maintenance of axonal excitability relies on coordination of multiple accommodating factors (e.g., ion channels).
- Neurophysiologic effects of ion channel modulating drugs ($BaCl_2$ and ivabradine) were monitored *in vivo* and quantified by computer modeling, suggesting secondary accommodation.
- Modification of single ion channels might not achieve its intended neurophysiologic effects due to accommodation by other ion channels.

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Two medicines were used for inhibition of ion channels : 1) for K^+ channel barium chloride ($BaCl_2$) (Wako, Japan) and 2) for hyperpolarization-activated, cyclic nucleotide-gated cation (HCN) channel ivabradine hydrochloride (Tokyo Chemical Industry, Japan) were used. For administration of medicines, $BaCl_2$ (9 mM) was dissolved in distilled water at the concentration of 2 mg/ml. $BaCl_2$ solution (20 mg/kg) was administered intraperitoneally in 7 male mice. The dosing was determined arbitrarily at the 40% of the reported LD50 by acute administration of mice (51 mg/kg) (3). Ivabradine hydrochloride, a selectively inhibiting agent of HCN channels, was dissolved in water at the concentration of 1 mg/0.5 ml. The solution of ivabradine (20 mg/kg) was administered intraperitoneally in 5 male mice, at which level anti-epileptic effect was reported in mice model (4).

Axonal excitability study. Electrophysiological study setup and neuronal excitability recording (TRONDNF protocol) were performed as previously explained in detail (5). A set of excitability parameters was derived from the recordings as previously described (6), namely threshold electrotonus (TE) ; the recovery cycle (RC) ; the current-threshold relationship (I/V) ; the strength-duration time constant (SDTC). One cycle of the multiple excitability tests takes approximately 20 minutes.

The baseline recording of axonal excitability was undertaken and axonal excitability testing was recorded again 45 minutes post-injection. For studying a low dose response, a separate study was performed with dividing the doses of the drugs and provided repeatedly as same manner.

Statistical analysis. The axonal excitability testing parameter changes before and after a single drug concentration was assessed with a paired t test using SPSS version 20 software (IBM, New York, USA) where a significance was set at $P< 0.05$.

Mathematical modeling. To provide insight into the membrane parameters underlying changes seen in the effect of an inhibitor, the Bostock model of the motor axon was used in the simulation of axonal excitability (MEMFit, QtracP version 01/03/2015), as previously explained in detail (7-9). To reflect better the characteristic waveform changes, the weighting factors were set as follows : TE, 3 ; RC, 1 ; SDTC, 1 ; and I/V, 3. The following tested parameters were adjusted to minimize the mean error for the simulated waveforms to best reproduce the average of the recorded waveforms : nodal and internodal resting potentials, nodal sodium permeability, percent persistent Na^+, nodal and internodal slow K^+ conductance, nodal and internodal fast K^+ conductance, internodal H conductance, nodal and internodal leak conductance, Barrett-Barrett conductance, and total pump currents.

RESULTS

Excitability study

Inhibition by $BaCl_2$. A comparison of nerve excitability parameters between at baseline and after administration of $BaCl_2$ was shown in Figure 1 and Table 1. The following interval changes were noted :

1) The current-threshold relationship (I/V) (Figure 1, Panel A) showed a significant reduction of resting slope ($P=0.02$).

2) In TE (Figure 1, Panel B), greater threshold changes by both depolarizing and hyperpolarizing currents were present. S2 accommodation that reflects the nodal slow K^+ current activity was decreased at post-injection ($P=0.005$).

3) RC measurements (Figure 1, Panel C), Refractoriness was decreased after injection ($P=0.03$). The superexcitability and late subexcitability tended to be smaller ($P=0.002$ and 0.1, respectively).

4) SDTC (Figure 1, Panel D) tended to become slightly smaller at post-injection ($P=0.2$).

5) A separate study administering $BaCl_2$ at two divided doses was performed in a single mouse. A small amount (4 mg/kg) of $BaCl_2$ resulted in the following : (1) decreased threshold reduction by long hyperpolarization (i.e., downward shift of the waveform) and slight reduction of late subexcitability and (2) unchanged S2 accommodation. Additional administration of $BaCl_2$ 30 minutes apart (total dosage : 20 mg/kg) resulted in decrease of S2 accommodation and late subexcitability (Figure 2, panels A-B), suggesting simultaneous effects on slow K^+ currents and Ih, instead of specific effects on slow K^+ current, followed by nonspecific action on Ih (see discussion).

Inhibition by ivabradine. Nerve excitability parameters changing by ivabradine was illustrated in Figure 3. The following changes were noted after administration of ivabradine in comparison to baseline values (Table 2) :

1) The response for the stimulation was increased ($P=0.04$) after the administration of ivabradine in the stimulus-current relationship.

2) The current-threshold relationship (I/V) showed smaller resting I/V slope ($P=0.009$), but no significant difference with depolarizing and hyperpolarizing currents.

3) In TE, hyperpolarizing current demonstrated significant greater threshold change, more so in the initial 40 ms. By strong hyperpolarization, TEh (peak,-70%) was greater after the drug administration ($P=0.02$). S3 accommodation induced by hyperpolarizing current increased with ivabradine ($P=0.008$). Threshold changes by depolarizing currents became greater by ivabradine, more so after 40 ms of stimulation.

4) In RC, late subexcitability tended to be smaller after ivabradine. Otherwise, no interval change was present.

5) SDTC showed no significant change by ivabradine.

6) Similar to $BaCl_2$, a study was performed to administer ivabradine at two divided doses in a single mouse. After administering the low dose (4 mg/kg), there was increase in threshold by long hyperpolarization (i.e., downward shift) as well as decreased late subexcitability. The downward shift of the hyperpolarizing TE was greater after administering the second dose 30 minutes after the initial injection (total dose : 20 mg/kg) (Figure 2). This suggests simultaneous blockage of Ih and slow K^+ current by ivabradine, instead of specific suppression of Ih followed by nonspecific suppression of slow K^+ currents (see discussion).

Modeling. The multiple axonal excitability data for the each group were averaged and modeled.

$BaCl_2$. Optimized parameters were automatically changed to minimize the error between the modelled and the observed waveforms. By changing single parameter, the post-administration waveforms were best fit by decreasing the internodal H conductance (GH), followed by decreasing slow potassium conductances (GKs), but the degrees of discrepancy reduction was marginal (an arbitrary goal was > 80% reduction). In order to achieve better discrepancy reduction, two parameters were adjusted and showed the best-fit by decreasing GH and GKs (Table 3). Further reduction was not achieved by increasing the parameters to three.

Ivabradine. Similar to $BaCl_2$, parameters were adjusted automatically to best fit the observed waveforms from the baseline recording. By 1-parameter scheme, decreasing GH showed the best discrepancy reduction, but the degree was not satisfactory. Thus two parameters were adjusted. The combination that showed the best discrepancy reduction was decreasing GH and 10-fold increasing the pump current, which was practically unrealistic and was rejected. The second-best combination was by decreasing GH by 80% and increasing the fast potassium conductances, followed by decreasing GH and increasing the slow potassium conductances. Further reduction was not achieved by increasing the parameters to three. The interval waveform changes by drug administration were not adequately reproduced by merely decreasing the target ion currents (Figure 4).

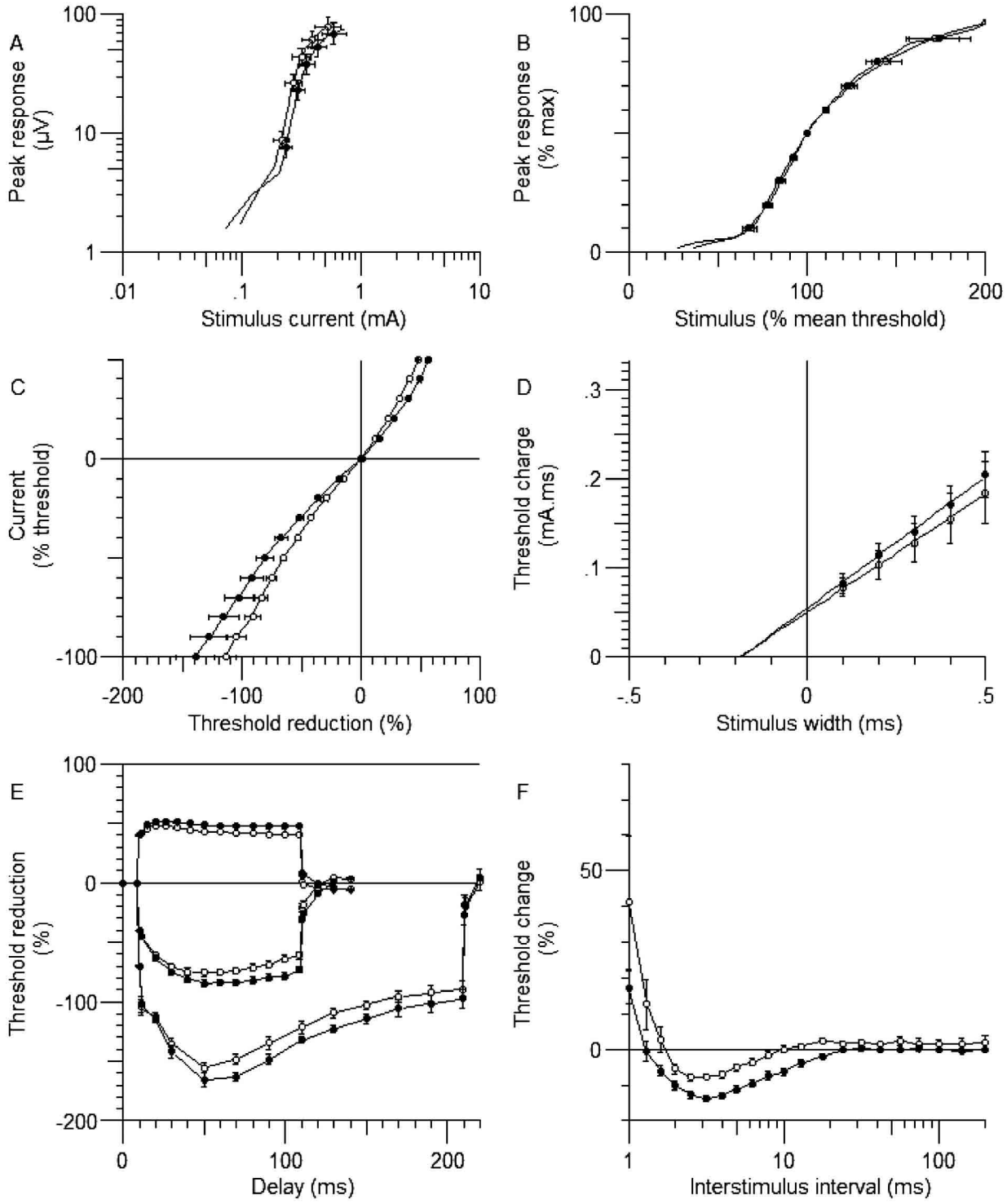

Figure 1 : Interval changes of axonal excitability by administration of $BaCl_2$. In comparison to the control waveforms (open circles), the post-administration waveforms show the following interval changes : (1) similar absolute and relative excitability by stimulus-response curves (Panels A and B), (2) outward shift of the current-threshold relationship (I/V) by long depolarizing and hyperpolarizing conditioning currents (Panels C and E), (3) decreased accommodation to long depolarization (S2 accommodation, Panel E) and late subexcitability (Panel F).

DISCUSSION

In the present study, we assessed interval changes of sensory axonal excitability *in vivo* by administering an ion channel inhibitor against K^+ channels or HCN channels. In addition to the expected inhibitory effects on the target channels, each inhibitor showed complex functional changes, suggesting accommodation by other channels. We assume that this compensatory mechanisms are important in stabilizing axonal excitability from external changes and have clinical implications on pharmacotherapy.

Table 1. Comparison of nerve excitability parameters at the baseline and after administration of $BaCl_2$ (N=7) (mean±SEM)

	Pre-injection	Post-injection	*P* values
Stimulus/current relationship			
Stimulus for max response (50%) (mA)	0.3±0.2	0.3±0.2	0.8
Peak response (μV)	87.9±1.2	79.5±1.2	0.7
Threshold electrotonus			
TEd (10-20 ms)	47.8±0.5	51.1±0.7	0.002
TEd (40-60 ms)	42.6±0.8	47.8±1.3	0.003
TEd (90-100 ms)	41.0±0.6	47.3±1.4	0.001
TEh (10-20 ms)	-65.9±2.2	-69.3±2.2	0.4
TEh (20-40 ms)	-74.1±2.8	-80.4±2.8	0.2
TEh (90-100 ms)	-62.7±3.2	-75.7±2.3	0.012
S2 accommodation	6.3±0.4	3.6±0.7	0.005
TEh (peak,-70%)	-153.5±4.4	-166.3±4.4	0.08
S3 accommodation	62.2±8.2	66.8±11.1	0.8
Recovery cycle			
Refractoriness (2.5 ms) (%)	-7.9±0.8	-12.7±1.0	0.03
Relative refractory period	1.6±1.1	1.3±1.0	0.2
Superexcitability (%)	-8.2±0.4	-13.4±0.8	0.002
Late subexcitability (%)	2.9±1.2	0.9±0.2	0.1
Current/threshold relationship			
Resting I/V slope	0.77±0.03	0.62±0.05	0.02
Minimum I/V slope	0.63±0.02	0.52±0.03	0.005
Hyperpolarizing I/V slope	0.96±0.13	0.94±0.10	0.9
Strength-duration time property			
Strength-duration time constant	0.23±0.03	0.19±0.02	0.2

Possible mechanisms for accommodation

In the present study, we administered two drugs that are well known to be ion channel blockers, $BaCl_2$ on K channels and ivabradine on HCN channels (10-12). The similar crystal radius of Ba^{2+} compared with K^+ and its stronger charge allows Ba^{2+} to fit tightly into the deep selectivity filter of K^+ channels, acting as K^+ channel inhibitor including Kv7, Shaker, and inward rectifier channels (13, 14). Barium itself has very weak inhibitory function of HCN channels (15). Ivabradine is a selective inhibitor of HCN channels, especially on HCN1 subunit and HCN4 (prominently expressed in the pacemaker region of the myocardium). Neither of the agents has been reported to act significantly on INaP or transient Na^+ current. The present study demonstrating complex excitability changes by these drugs, thus suggests altered functions of the channels other than their primary targets.

After administration of barium, there was reduction of Ih and possibly INaP, along with expected reduction of fast and slow K^+ currents. Blockade of IKs causes hyperexcitability, manifesting as seizure and muscle cramps (16, 17).

On the other hand, ivabradine significantly suppressed Ih as expected, as well as increase of either fast or slow K^+ currents. Although there were contradictory results in Table 4 (decrease of GKf and GKs by changing one parameter and vice versa by changing two parameters), the decreases by one-parameter format did not yield satisfactory discrepancy reduction and thus do not fully explain the waveform changes. By suppressing excitatory Ih by ivabradine, axonal excitability becomes decreased. Ih has a negative-feedback property ; if hyperpolarizing current is applied, Ih is activated and permeability of Na^+ increases, resulting in returning membrane potential toward rest. On the other hand, if depolarizing current is applied, Ih that is active at rest becomes deactivated, again returning membrane potential at rest (18). Blocking Ih hyperpolarizes the membrane, whereas the input resistance increases (19), that results in a delicate conductance-current balance. To summarize, Ih embodies two opposing influences on neuronal excitability and makes simple characterization as either inhibitory or excitatory to be difficult. It is intriguing that in the central nervous system, inhibitory action of Ih is caused by its interaction with delayed rectifier M-type K^+ current (20), but we are not aware of such report in the peripheral nerve. The present data complement our previous report of acute effects of blocking INaP by ranolazine (21), that showed features suggestive of suppression of INaP, transient Na^+ current, and slow K^+ currents.

Whether resting potential could be affected by either barium or ivabradine is not straightforward. Excitability parameters that are sensitive to axonal membrane potentials are the following (22) : (1) superexcitability, (2) the resting current-threshold slope, (3) depolarizing and hyperpolarizing threshold electrotonus at 90-100 ms, and (4) subexcitability. However, 1, 3, and 4 are affected by either decreasing slow K^+ conductance or Ih (Figure 3). Decrease of the resting current-threshold slope by both barium and ivabradine thus suggests hyperpolarizing shift of the resting potential, but it was not fully supported by other parameters.

Clinical significance

Because neuronal ion channels have been identified as important pathophysiological and therapeutic targets in many neurological conditions, ion-channel modifying drugs are one of the main therapies on these conditions. However, monitoring the channel functions *in vivo* by the particular drug in the target organ is not straightforward, especially so in the nervous system. As discussed above, because fine regulation of neuronal excitability relies on complex interaction involving multiple ion channels and their

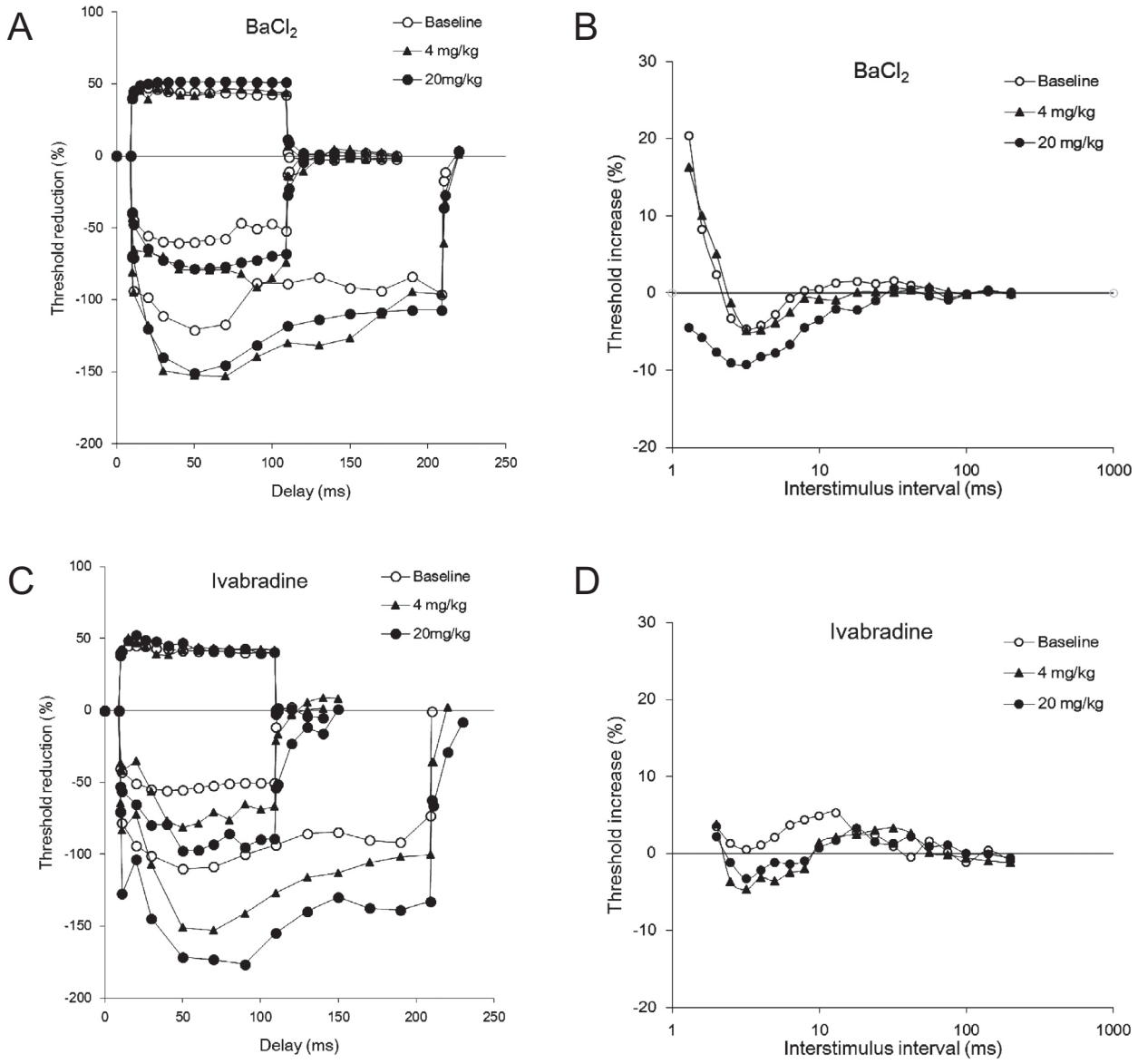

Figure 2 : A separate study administering $BaCl_2$ at two divided doses in a single mouse. A small amount (4 mg/kg) of $BaCl_2$ resulted in the following : (1) decreased threshold reduction by long hyperpolarization and slight reduction of late subexcitability, but unchanged S2 accommodation. Additional administration of $BaCl_2$ 30 minutes apart (total dosage : 20 mg/kg) resulted in decrease of S2 accommodation and late subexcitability (Figure 2, panels A-B), suggesting simultaneous effects on slow K^+ currents and Ih.
Ivabradine was similarly administered at two divided doses in a single mouse. After administering the low dose (4 mg/kg), there was increase in threshold by long hyperpolarization as well as decreased late subexcitability. The downward shift of the hyperpolarizing TE was greater after at the total dose of 20 mg/kg (Figure 2, panels C-D). This suggests simultaneous blockage of Ih and slow K^+ current by ivabradine.

modulators, *in vivo* effect of a channel-modifying agent might not be identical to observed results in a simpler *in vitro* system or computer simulation. Threshold tracking is a non-invasive method to quantify multiple ion channel functions *in vivo*, thus it has advantage of depicting overall excitability as well as behavior of specific ion channels in a dynamic manner, certainly in a clinical setting. This report has implication to therapeutic use of channel-modifying agents in neurological diseases, that is, to possible effects on multiple, often unexpected, ion channels and overall excitability.

Limitation

This study has limitations. First, the serum concentrations of the medications were not measured. However, correlation between the drug concentrations in the serum and the peripheral nerves may not be linear, thus measurement of serum concentrations might not directly reflect pharmacological effect in the peripheral nervous system. Second, suppression of Ih by ivabradine could have been only partially evaluated because anesthesia by isoflurane also suppresses Ih even at pre-administrative stage (5). It may partially explain lower discrepancy reduction by the modeling study by ivabradine (Table 4). Third, modeling study cannot specify responsible parameters exactly because no single parameter or a combination of parameters stand out. Another limitation on modeling is unrealistic calculation, as seen in Table 4 with 10-fold increase of pump current.

In summary, we underwent *in vivo* axonal excitability study in normal mice to identify interval changes of multiple ion channel functions by administration of specific channel blockers, $BaCl_2$ and

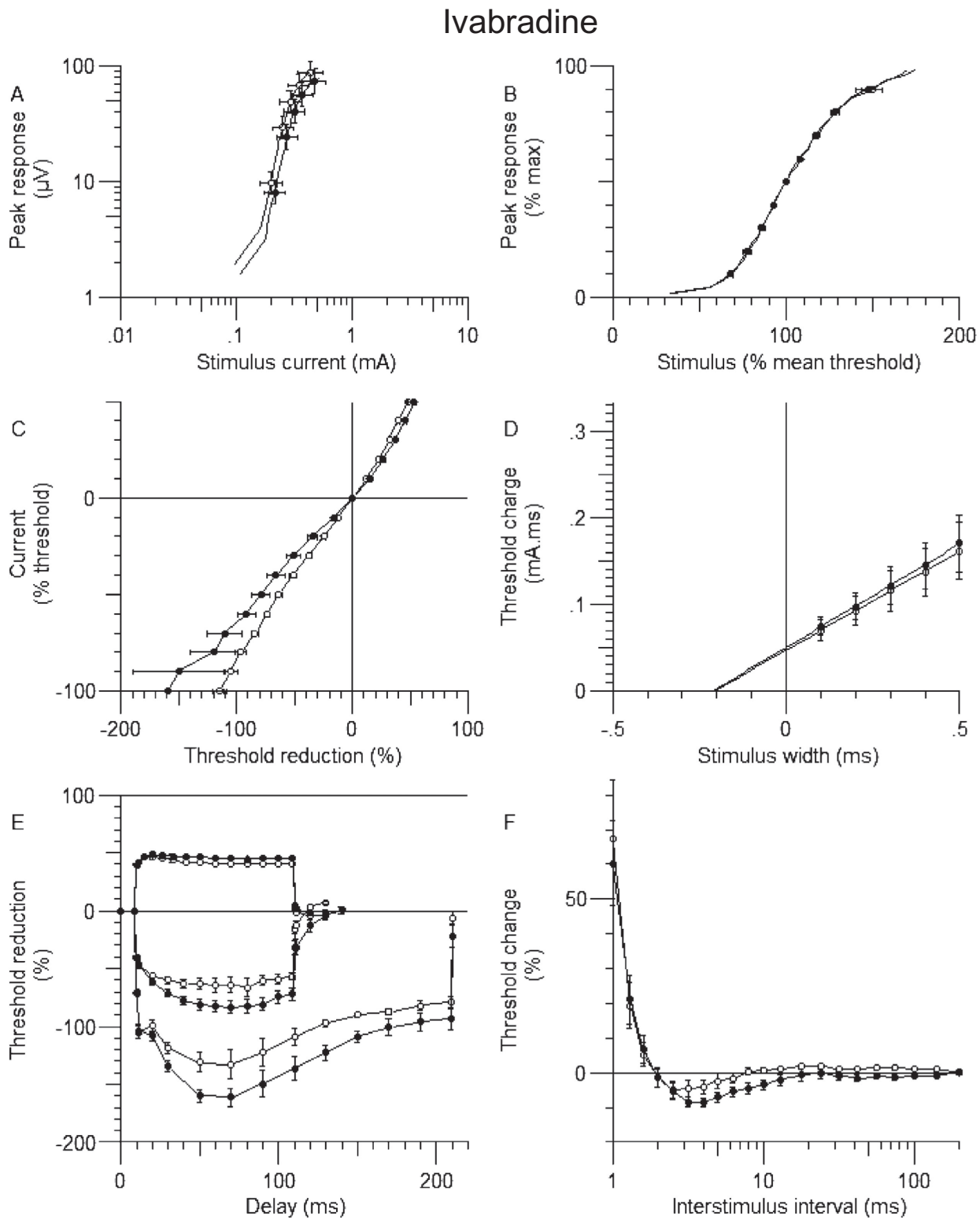

Figure 3 : Interval changes of axonal excitability by administration of ivabradine. In comparison to the control waveforms (open circles), the post-administration waveforms show the following interval changes : (1) similar stimulus-response curves, both by absolute and relative scales (Panels A and B), (2) outward shift of the current-threshold relationship (I/V) by long hyperpolarizing and depolarizing currents (Panels C and E), (3) decreased accommodation to long depolarization (S2 accommodation, Panel E) and late subexcitability (Panel F). Overall, these changes are similar to those seen by administration of $BaCl_2$ (Figure 1).

Table 2. Comparison of nerve excitability parameters at the baseline and after administration of ivabradine (N=5) (mean±SEM)

	Pre-injection	Post-injection	*P* values
Stimulus/current relationship			
Stimulus for max response (50%) (mA)	0.32±0.15	0.34±0.16	0.04
Peak response (μV)	98.0±1.3	81.2±1.3	0.003
Threshold electrotonus			
TEd (10-20 ms)	45.6±1.7	48.2±1.3	0.09
TEd (40-60 ms)	41.0±1.0	45.5±0.9	0.01
TEd (90-100 ms)	40.9±1.1	45.1±0.8	0.008
TEh (10-20 ms)	-58.3 ±1.7	-67.1±2.6	0.004
TEh (20-40 ms)	-62.4±3.7	-77.2±3.6	0.001
TEh (90-100 ms)	-57.9±3.6	-73.3±5.4	0.055
S2 accommodation	4.6±1.0	3.1±1.1	0.3
TEh (peak,-70%)	-137.7±9.2	-162.0±6.9	0.02
S3 accommodation	57.2±13.7	68.0±11.7	0.008
Recovery cycle			
Refractoriness (2.5 ms) (%)	-5.1±2.6	-5.2±2.2	0.9
Relative refractory period	1.7±1.1	1.9±1.1	0.6
Superexcitability (%)	-5.0±2.0	-8.0±1.33	0.2
Late subexcitability (%)	2.1±0.2	1.1±0.4	0.13
Current/threshold relationship			
Resting I/V slope	0.85±0.07	0.66±0.07	0.009
Minimum I/V slope	0.68±0.04	0.49±0.09	0.1
Hyperpolarizing I/V slope	1.19±0.22	0.77±0.17	0.2
Strength-duration time property			
Strength-duration time constant	0.22±0.03	0.25±0.05	0.4

Table 3 : Modeling the nerve excitability data from animals post-administration of $BaCl_2$ (discrepancy reduction from pre-administration waveforms/parameter settings)

	Parameter	change	discrepancy reduction
1	GH	-66%	77.9%
2	GKs	-87%	73.7%
3	GKf	-86%	54.6%
4	GBB	+38%	43.2%
5	GLk	-41%	40.7%

	Parameter 1	change	Parameter 2	change	discrepancy reduction
1	GH	-52%	GKs	-40%	82.4%
2	PNap (%)	-46%	GH	-66%	79.2%
3	GKs	-77%	GLk	-18%	78.8%
4	GH	-64%	GKf	-2%	78.6%
5	GBB	+13%	GKs	-86%	78.5%

GBB='Barrett-Barrett' conductance across myelin sheath ; GH=internodal H conductance ; GKs=nodal and internodal slow potassium conductances ; GLk=nodal and internodal leak conductances ; PNap=persistent sodium conductance (as% of nodal sodium permeability)

ivabradine. The data suggest that modification of functions in other than the target channels. This information could be useful in planning ion-channel targeting therapy in neurological diseases.

ABBREVIATIONS

HCN channel, hyperpolarization-activated cyclic nucleotide-gated channel
Ih, hyperpolarization-activated cation current
IKs, slow K^+ current
INaP, persistent Na^+ current
IV, current-threshold relationship
RC, recovery cycle
SDTC, strength-duration time constant
TE, threshold electrotonus.

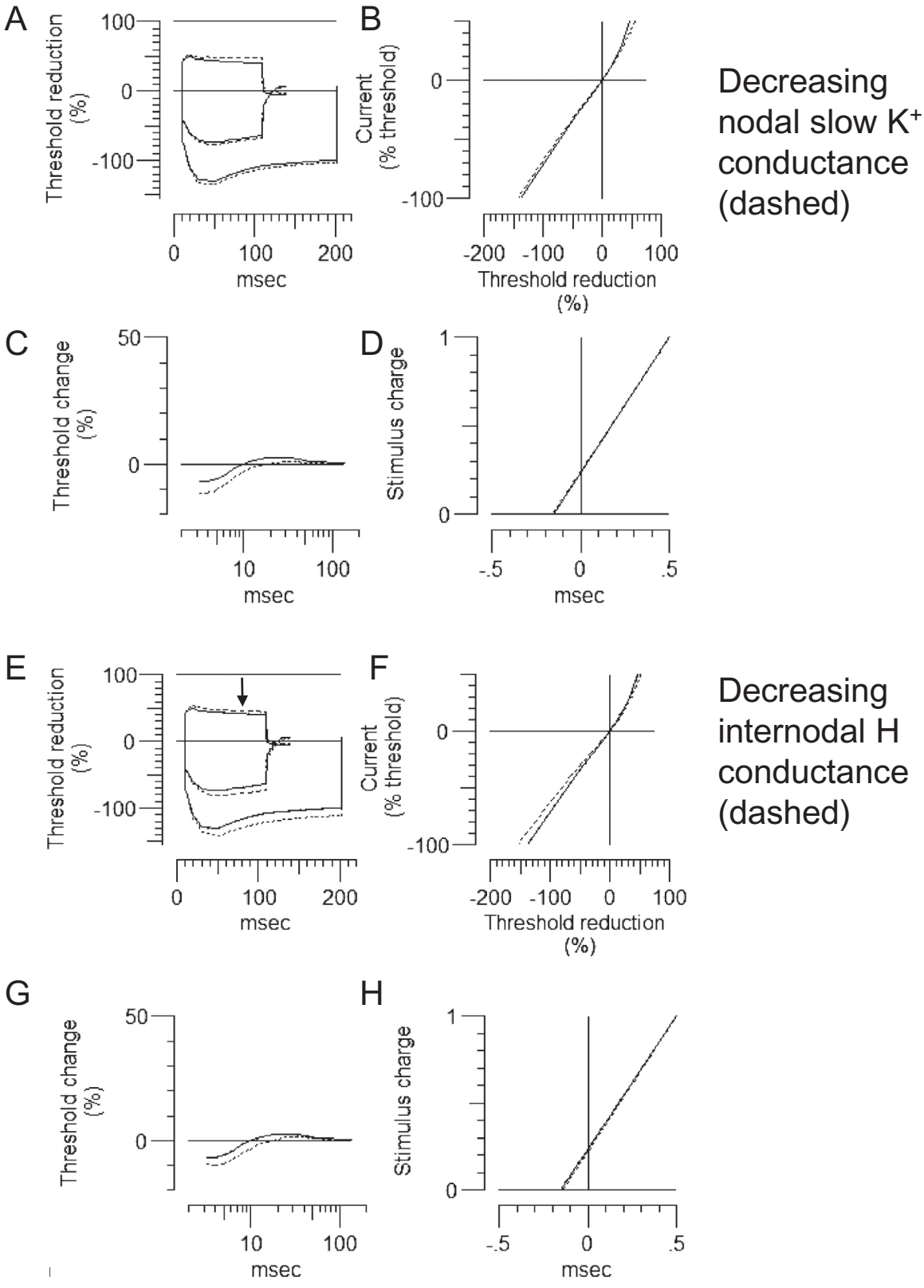

Figure 4 : Modeled waveform changes by altering 1 parameter. (1) By arbitrarily decreasing the nodal slow K^+ conductance (GKsN) to 25% of normal, accommodation to long depolarizing current is lost (Panels A and B) and there are decreased late subexcitability and increased supernormality (Panel C). (2) By arbitrarily decreasing the internodal H conductance (GH) to 50% of normal, there are increased threshold changes to hyperpolarizing current (Panels E and F) and decreased late subexcitability and increased supernormality (Panel G). Of note, accommodation to depolarizing current was unchanged (arrow, Panel E). These changes do not reproduce the waveforms by administering $BaCl_2$ or ivabradine (Figures 1-2), suggesting alteration of multiple ion channel properties by the respective compounds.

Table 4 : Modeling of the nerve excitability data from animals post-administration of ivabradine (discrepancy reduction from pre-administration waveforms)

	Parameter	change	discrepancy reduction
1	GH	-58%	63.0%
2	GKf	-71%	44.6%
3	GKs	-99%	41.5%
4	PNap	+4800%	36.6%
5	PNaN	+327%	35.2%

	Parameter 1	change	Parameter 2	change	discrepancy reduction
1	GH	-77%	IPumpNI	+1000%	81.5%
2	GH	-82%	GKf	+244%	73.9%
3	GH	-83%	GKs	+228%	68.3%
4	PNaN	-41%	GH	-71%	67.5%
5	GH	-53%	GBB	+29%	65.6%

GBB = 'Barrett-Barrett' conductance across myelin sheath ; GH = internodal H conductance ; GKf = nodal and internodal fast potassium conductances ; GKs = nodal and internodal slow potassium conductances ; GLk = nodal and internodal leak conductances ; PNaN = nodal sodium permeability ; PNap = persistent sodium conductance (as % of nodal sodium permeability)

CONFLICT OF INTEREST STATEMENT

None of the authors has conflict of interest.

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